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(54) Title: A METHOD FOR INTRODUCING MOLECULES, PARTICULARLY GENETIC MATERIAL, INTO PLANT CELLS

(57) Abstract

A method for introducing molecules, particularly genetic material, into intact plant cells, whereby a medium comprising said plant cells and said molecules is subjected to mild ultrasound treatment.

* See back of page

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Title: A method for introducing molecules, particularly genetic material, into plant cells

## Technical Field

The present invention relates to a method for introducing molecules, particularly genetic material, into intact plant cells.

## Background Art

Several methods are known for introducing molecules, such as genetic material, for instance plasmid DNA, RNA, vira or fragments thereof, into animal cells and protoplasts.

Such known methods include inter alia chemical methods, electroporation and microinjection. It has been shown that such known methods could be implemented in both transient and stable transformation. In general all methods disclosed for obtaining transient expression have turned out to be usable for obtaining stable transformation.

Concerning the introduction of genetic material, plant protoplasts and animal cells have the one important common feature that both are separated from the environment by a plasma membrane only. Intact plant cells, on the other hand, do not only have a plasma membrane but also a cell wall impenetrable for high molecular compounds, said cell wall being a tight network of cellulose fibrilles, pectin and often also lignin.

The similarity between plant protoplasts and animal cells is further emphasized by the fact that known methods for introducing genetic material into plant protoplasts can usually also be used for animal cells.

Such methods compris :

Calcium phosphate precipitation of plasmid DNA with formation of a crystalline product. This product can be absorbed by animal cells (Graham and van der Eb, Virology, 52, 456-457, 1973) and by plant protoplasts (Hain et al., Mol. Gen. Genet., 199, 161-168, 1985).

Encapsulation of plasmid DNA with liposomes and subsequent fusion with plant protoplasts (Deshayes et al., EMBO, 4, 2731-2737, 1985) or with animal cells (Felgner et al., Proc. Natl. Acad. Sci. USA, 84, 7413, 1987), said fusion being induced by polyethylene glycol.

Use of polyethylene glycol (PEG). In this method polyethylene glycol, usually 40% polyethylene glycol 6000, is added to plant protoplasts and genetic material (Krens et al., Nature, 296, 72-74, 1982). Polyethylene imine or poly-L-ornithin can be used correspondingly.

Electroporation. In this case a suspension of animal cells or plant protoplasts is exposed to a short electric pulse of high field strength in the presence of genetic material (Neuman et al., EMBO J., 1, 841-845, 1982; Fromm et al., Nature, 319, 791-793, 1986).

Microinjection. In this case the genetic material is introduced into plant protoplasts (Crossway et al., Mol. Gen. Genet., 20, 179, 1986) or into animal cells (Cappechi, Cell, 22, 479-488, 1980) by means of an ultra-fine micropipette.

WO Publication N . 89/02464 disclos s th transf rmati n of animal cells with DNA fragments by subjecting the cells to ultrasound treatment sufficient to traumatize the cells without killing them. Due to the similarities between animal cells and plant protoplasts mentioned above it has to be considered obvious that this method can also be used to introduce DNA fragments in plant protoplasts. Since none of the above-mentioned methods turned out to be suitable for introducing plasmid DNA into intact plant cells, a person skilled in the art reading the WO publication will come to the conclusion that this method can probably also be implemented with protoplasts. He will, however, hardly consider using said method for intact plant cells difficult to penetrate. It is thus generally accepted among those skilled in the art that the walls of plant cells are in general impenetrable for larger molecules such as DNA and proteins.

To solve this problem it was therefore necessary to first remove the cell walls of the plant cells, usually by enzymatic hydrolysis, to subsequently introduce genetic material in the resulting protoplasts. Usually this causes difficulties, as it is normally even more difficult to regenerate an entire plant from protoplasts than from intact plant cells. There is thus a great need to find a method to introduce genetic material directly into intact plant cells to avoid the problems connected with creating protoplasts as well as problems connected with regeneration from protoplasts.

Recently, a method was developed to introduce plasmid DNA into intact plant cells. This method is based on bombarding intact plant cells with small particles coated with plasmid DNA at high velocity (Klein et al., Nature, 327, 70-73, 1987). This meth d, however, requires v ry

expensive equipment which in turn demands consid rabl expert knowledge to be operated correctly.

There is thus a need for a more simple and inexpensive method for introducing molecules, particularly genetic material, into intact plant cells.

It has been found that the introduction of molecules, particularly genetic material, into intact plant cells can be achieved by means of mild ultrasound treatment using a method requiring comparatively inexpensive and easily available equipment while being quite effective.

Description of Invention

The object of the present invention is to provide a method for introducing molecules, particularly genetic material, into intact plant cells, in an inexpensive and effective way.

This is accomplished by the method according to the invention, characterized by a medium comprising said plant cells and said molecules being subjected to mild ultrasound treatment.

The method according to the invention is a useful alternative to previously known introduction methods. The method provides a new approach involving only moderate costs, said method being carried out quickly and simply. Based on introduction experiments already conducted it can be predicted that the method will also turn out to be superior when used for a great number of other biological materials, where known methods are insufficient.

According to the inventiv m thod th plant cells are subjected to an ultrasound treatment considerably milder

than th ultras und treatm nt used f r homog nizing or lysing cells. It is important that the treatment is suitably mild so that a sufficient number of plant cells remain viable. To ensure viability the plant cell suspension can advantageously be exposed to ultrasonic waves of a frequency range of from 5 kHz to 10 MHz, particularly from 10 to 100 kHz, and an electric output power (i.e. the electric output power as read from the "output control" of the electric power supply unit and supplied to the sound-emitting means) of up to 600 W, such as from 5 to 300 W, preferably from 30 to 90 W, for a period of up to 10.000 ms, such as from 100 to 3.000 ms, preferably from 400 to 1.000 ms.

Examples of molecules introducable into plant cells by the method according to the invention include DNA, plasmid DNA, RNA, vira, proteins, lipids, pharmaceutical compositions, small molecules, organelles or fragments of such materials.

The method according to the invention has advantageously been used to introduce molecules into cells of sugar beet and tobacco plants.

When introducing genetic material, such as plasmid DNA, into plant cells of sugar beets and tobacco, particularly good results are obtained by using ultrasonic waves of a frequency range of from 10 to 30 kHz at an electric output power of from 75 to 100 W for a period of from 500 to 1.000 ms.

A particularly suitable medium for plant cells and molecules during ultrasound treatment is CPW comprising from 21 to 28% sucrose.

Wh n introducing plasmid DNA by the method according to the invention, particularly favourable results are

btained, when th concentrati n of plasmid DNA in the medium is at least 10 µg/ml.

The mild ultrasound treatment of the method according to the invention is advantageously carried out using a sound-emitting means having an acute point, said means being only immersed in the upper portion of the medium.

It was shown that molecules have been introduced into plant cells of dicotyledons such as sugar beet and tobacco plants by means of the method according to the invention. Presumably the method is just as suitable for introducing molecules into plant cells of monocotyledons.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The method according to the invention is advantageously carried out by using a device comprising a sound-emitting means capable of emitting ultrasound in a medium for a period of up to 10.000 ms. Such a device is advantageously provided in such a way that it emits ultrasonic waves of a frequency in the range of from 10 to 100 kHz.

To perform a variety of tasks the device according to the invention can be equipped so that the electric power supplied to th sound-emitting means can be adjusted to any given valu in the rang of from 5 to 300 W and so

that th duration f the ultrasou:d tr atment can be adjusted within a range of from 100 to 10.000 ms.

The sound source of the device is advantageously formed in such a way that said device can be immersed in a medium in a suitable vessel, for instance an Eppendorf tube. Such an embodiment, where the sound-emitting means is situated at the end of a thin rod, is known from conventional ultrasound devices for degrading or lysing cell material.

Experiments have shown that such conventional ultrasound devices are usable for the method according to the invention. The present invention has been developed using a device for lysing cells, said device being commercially available from Branson, Eagle Road, Danbury, Connecticut, USA, under the name of Sonifier B 15. The device can emit ultrasound of a frequency of 20 kHz. The electric power values expressed in watts mentioned in the present specification with claims are electric output powers as read from the output control of the electric power supply unit. During experiments the ultrasound-emitting means was immersed for approx. 2 to 3 mm measured from the surface. Preliminary experiments with a calorimeter have shown that the ultrasonic power imparted to the liquid during experimental procedure is approx. 5 to 10% of the given electric output power. When other devices are used, a person skilled in the art is able to determine a suitable adjustment in the same way as described in the examples hereinafter, said adjustment resulting in a mild ultrasound treatment and an effective introduction of molecules, while maintaining sufficient viability. In other words the adjustment results in a power of the ultrasound treatment corresponding to that of the experiments performed according to the examples. For comparativ purposes it may be noted that when th device mention d, Sonifi r B 15, is used to lys or homog nize

cells under otherwise the same conditi ns, it is adjusted to an electric output power of from 80 to 100 W and a treatment period of from 30.000 to 250.000 ms when treating intact cells.

As mentioned above, several methods for introducing genetic material into plant cells are known, where said plant cells are first transformed to plant protoplasts, as the amount of introduced genetic material into protoplasts is higher, since said protoplasts do not have cell wall barriers. On the other hand, it is difficult to regenerate protoplasts of a number of plant species, particularly monocotyledons, to entire plants. Although favourable results have been obtained using protoplasts of certain plant species, there are other plant species, where it will be more suitable to use intact plant cells, plant embryos or other morphogenous material instead of protoplasts. Experiments have shown that it is possible and advantageous to introduce genetic material directly into intact cells using mild ultrasound treatment by means of the method according to the invention, as this is a less complicated method allowing a considerable increase in the number of plant species successfully submitted to genetic manipulation.

The genetic material in question is especially DNA or fragments thereof, such as plasmid DNA. Presumably the method is also suitable for introducing RNA or fragments thereof as well as vira, for instance for pathological tests. The method according to the invention is probably also suitable for introducing proteins, lipids, pharmaceutical compositions, small molecules as well as organelles and virus particles into cells.

Th medium can be any suitabl , c nventi nal medium f r cells or protoplasts.

The method according to the invention uses a technique presumbably resulting in a temporary moderate weakening of both the cell membrane and the cell wall, which is shown in intact plant cells in the following examples. The examples thus show that plasmid DNA can penetrate plant cells having been treated with ultrasound.

## Best Mode for Carrying out Invention

## Examples

### General experimental conditions

### Cell suspension culture

A cell suspension culture of sugar beets (Beta vulgaris L.) of the genotype M1 (available from DANISCO A/S, Copenhagen, Denmark) is prepared from callus obtained from embryos. The cell suspension is cultured in darkness at 25°C on a rotary shaking table. The cells are maintained by sub-culturing in a medium according to Murashige and Skoog (Physiol. Plant., 15, 473-497, 1962) to which 5.7 µM indol acetic acid and 4.4 µM benzyladenine was added.

### CPW

CPW is an aqueous solution of a mixture of inorganic salts comprising i.a. approx. 10 mM $Ca^{++}$, described by Frearson et al., (Dev. Biol., 33, 130-137, 1973).

### Ultrasound treatment

A cell suspension for ultrasound treatment is prepared by removing cells 3 to 4 days after sub-culturing and washing them twice with CPW 13S (i.e. CPW containing 13% sorbitol), finally suspending said cells in CPW 13S at a

rati f 1 part by volume c lls t 4 parts by volume CPW 13S. Then plasmid DNA is added to the suspension in 0.35 ml CPW containing 21% sucrose and plant cells (500.000/ml) in an Eppendorf tube, the final plasmid concentration being 45 µg/ml. The plasmid DNA used is a plasmid coding for the marker enzyme chloramphenicol-acetyltransferase (CAT), in this case the plasmid pCaMVCN having the code 27-4909, available from Pharmacia LKB Biotechnology, Uppsala, Sweden.

Subsequent to quick shaking the microtip of a Sonifier B 15 (available from Branson, Eagle Road, Danbury, Connecticut, USA) is immersed in the upper half of the cell suspension (i.e. 2 to 3 mm measured from the surface). An ultrasound pulse of a frequency of 20 kHz is supplied. The power values mentioned in the experiments are determined on the basis of the output control, where one unit corresponds to 15 W electric power. At 10 W electric power the effective acoustic power measured is 0.22 W/cm$^2$. The duration of the treatment is adjusted by means of the scale for per cent duty circle, where an adjustment to 10% ensures that each ultrasound pulse has a duration of 110 ms. The number of such pulses of 110 ms can be controlled by the on/off switch.

The cells treated by ultrasound are then transferred to petri dishes containing a MS-medium (Murashige and Skoog, cf. above) and are incubated for 2 days at 23°C. The presence of CAT-activity is shown by adding $^{14}$C-marked chloramphenicol to an extract obtained from the treated cells, whereupon the sample is heated to 60°C for 6 min. After cooling acetyl-coenzyme A is added to the sample, the final concentration of acetyl-coenzyme A being 0.71 mM. The introduction of plasmids is assessed by measuring th percentage transf rmati n of chloramphenicol (CA). Th method used is a modification

f the method described by Gorman et al. (Mol. C ll. Biol., 2, 1044-1051, 1982).

Example 1

The present example illustrates the introduction of plasmid DNA into intact sugar beet cells of the genotype M1.

The suspension culture of sugar beet cells is maintained by sub-culturing as described above and is treated with ultrasound, cultured and analysed in the manner described. The results of the measured CAT-activity appear from Table 1.

Table 1
Sugar beets

| Power (Watt) | Time (ms) | Transformed CA (%) |
|---|---|---|
| 0 | 0 | 0.03 |
| 60 | 800 | 0.06 |
| 75 | 800 | 0.21 |
| 90 | 800 | 0.17 |
| 105 | 800 | 0.07 |

Example 2

The present example illustrates the introduction of plasmid DNA into intact tobacco cells.

A suspension culture of tobacco cells is maintained by sub-culturing in the manner described above for sub-culturing of cell suspensions with sugar beets, the culture medium, h wever, being a medium acc rding to Murashig and Sk g (Physiol. Plant. 15, 473-497, 1962),

to which were add d 0.2 mg/l 2,4-dichl rophenoxy acetic acid, 0.1 mg/l kinetin, 0.9 mg/l thiamin hydrochloride and 0.2 g/l $KH_2PO_4$, pH 6.0.

The cells are removed 3 to 4 days after sub-culturing and washed twice with CPW 13S (i.e. CPW containing 13% sorbitol), finally suspending said cells in CPW 13S at a ratio of 1 part by volume cells to 4 parts by volume CPW 13S. Samples of 0.35 ml each are taken out and the plasmid pCaMVCN is added to each sample, the final plasmid concentration being 100 µg/ml. The cells are then subjected to ultrasound treatment under the conditions appearing from Table 2. After culturing for 2 days in the above-mentioned medium the cells are extracted and their CAT-activity is measured. The results appear from Table 2.

Table 2

Tobacco

| Power (Watt) | Time (ms) | Transformed CA (%) |
|---|---|---|
| 0 | 0 | 0.05 |
| 75 | 570 | 0.06 |
| 75 | 800 | 0.13 |
| 75 | 1000 | 0.07 |

As is apparent the method according to the invention can be used to introduce molecules into intact plant cells.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scop of the invention, and all such modificati ns as

would be obvious to ne skilled in the art ar intend d to be included within the scope of the following claims.

Claims

1. A method for introducing molecules, particularly genetic material, into intact plant cells, c h a r a c t e r i s e d by a medium comprising said plant cells and said molecules being subjected to mild ultrasound treatment.

2. A method as claimed in claim 1, c h a r a c t e r i s e d by the mild ultrasound treatment being carried out by supplying a sound-emitting means with an electric power of up to 600 W over a period of up to 10.000 ms.

3. A method as claimed in claim 1, c h a r a c t e r i s e d by the mild ultrasound treatment being carried out by supplying the sound-emitting means with an electric power of from 5 to 300 W over a period of from 100 to 3000 ms.

4. A method as claimed in claim 1, c h a r a c t e r i s e d by the mild ultrasound treatment being carried out by supplying the sound-emitting means with an electric power of from 30 to 90 W over a period of from 400 to 1000 ms and by the frequency of the sound waves lying in the range of from 10 to 100 kHz.

5. A method as claimed in claim 1, c h a r a c t e r i s e d by the molecules being selected from a group comprising DNA, plasmid DNA, RNA, vira, proteins, lipids, pharmaceutical compositions, small molecules, organelles and fragments of such materials.

6. A method as claimed in claim 1, c h a r a c t e r i s e d by the concentration of plasmid DNA in the medium being at least 10 µg/ml.

7. A method as claimed in claim 1, c h a r a c t e r - i s e d by the mild ultrasound treatment being carried out using a sound-emitting means having an acute point, said means being only immersed in the upper portion of the medium.

8. A method as claimed in claim 1, c h a r a c t e r - i s e d by the plant cells being monocotyledons.

9. A method as claimed in claim 1, c h a r a c t e r - i s e d by the plant cells being dicotyledons.

10. A method as claimed in claim 9, c h a r a c t e r - i s e d by the plant cells being cells of sugar beet or tobacco plants.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00166

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/87, 15/64, A 01 H 1/00

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| IPC5 | C 12 N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category * | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| X | WO, A1, 8902464 (AMERSHAM INTERNATIONAL PLC) 23 March 1989, see the whole document -- | 1-10 |
| A | EP, A2, 0290395 (SANDOZ AG) 9 November 1988, see the whole document -- | 1-10 |
| A | DE, A1, 3537261 (GCA CORP.) 30 April 1987, see the whole document -- | 1-10 |
| A | Chemical Abstracts, volume 72, no. 17, 27 April 1970, (Columbus, Ohio, US), Lazaretnik, A. Sh. et al.: "Phonophoresis in conditions of experimental tumors and leucoses ", see page 187, abstract 88206s, & Fiziol. Zh. (KIEV) 1969, 15( 6), 8428- 46 -- | 1-10 |

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## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 1st October 1990 | 1990 -10- 0 5 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| SWEDISH PATENT OFFICE | Mikael Bergstrand |

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# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00166

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| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
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| EP-A2- 0290395 | 88-11-09 | AU-D- | 1550888 | 88-11-10 |
| | | JP-A- | 63301792 | 88-12-08 |
| DE-A1- 3537261 | 87-04-30 | JP-T- | 63501613 | 88-06-23 |
| | | WO-A- | 87/02383 | 87-04-23 |